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Review of HPLC and LC-MS/MS Methods for Determination of ZT-1 and Its Active Metabolite, Huperzine A in Various Biological Matrices

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Abstract

Huperzine A is an alkaloid isolated from *Huperzia serrata*. It belongs to anticholinesterase class and used for neurological conditions like Alzheimer's disease. Huperzine A is the metabolite of ZT-1, which is also used for the treatment of memory disorders and inhibits the effect of enzyme and brings back sufficient levels of acetylcholine. This review provides an overview of various HPLC, LC-MS and LC-MS/MS methods for quantitation of Huperzine A alone or along with ZT-1 in different biological fluids (plasma, serum and blood). In addition, this review provides complete details on sample preparation, extraction recoveries, matrix effect, internal standard selection, selectivity optimization, chromatographic separations, mass spectrometric spectrometry and other detection techniques etc.

Keywords: Huperzine A; ZT-1; HPLC; LC-MS/MS; Bioanalytical methods; Spectrometry

Introduction

Huperzine A is a novel sesquiterpene alkaloid isolated from *Huperzia serrata* [1,2] and it is also derived from other *Huperzia* species including *H. elmeri*, *H. carinat* and *H. aqualupian* [3]. Huperzine A (CAS No.: 102518-79-6; Figure 1), chemically [(5R,9R,11E)-5-amino-1-ethyldiene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocloocta-[B]-pyri-din-2(1H)-one] is a potent, selective and slow reversible Acetylcholinesterase (AChE) inhibitor [4,5], which has a mechanism of action similar to donepezil, rivastigmine and galantamine. Huperzine A readily crosses blood brain barrier and shows high specificity for AChE. By using X-ray crystallography it was confirmed that the complexity of huperzine A with AChE Crystal Structure of AChE complexed with huperzine A confirm the specificity of inhibitor [6]. The outcome of clinical trials showed that huperzine A capsule shows is efficient and safe to treat mild to moderate Alzheimer's disease and also enhance the memory and learning performance of adolescence students [7]. Huperzine A has been approved in China for the treatment of Alzheimer's disease (in the form of immediate release tablets and capsules) and vascular dementia without any notable side effects [8-12]. It is being marketed as a dietary supplement in USA [12]. Huperzine A is administered orally at a dose range of 0.2-0.4 mg/day for Alzheimer's disease and 0.06-0.4 mg/day (intramuscularly) for dementia. Following oral administration of 0.4 mg huperzine A tablets to healthy human volunteers peak plasma concentrations (C_{max}: 2.59 ng/mL ± 0.37 ng/mL) achieved at around 1 h (T_{max}). The mean α and β half-life was is 21.13 min ± 7.28 min and 716 min ± 130 min, respectively [13].

ZT-1, N-[2-hydroxy-3-methoxy-5-chlorobenzilidene] huperzine A is a novel AChE inhibitor, which rapidly transformed non-enzymatically into its active metabolite huperzine A. The dual mode of action of ZT-1 as an N-methyl-D-aspartate (NMDA) receptor antagonist and an AChE inhibitor, positions it as a third generation anti-Alzheimer's product by improving the general condition and cognitive functions of affected patients as well as having the potential of being a neuroprotectant [14].

Scope

The aim of this review is to provide: (a) A brief compilation of various analytical methodologies on HPLC, LC-MS and LC-MS/MS for the analysis of huperzine A and ZT-1 in plasma and other biological fluids, which we have captured in a tabular format. It contains sample processing, chromatographic conditions, validation parameters and applicable conclusions; (b) Discussed details about sample preparation, internal standard selection, matrix effect, chromatography and various detection systems. Tables 1 and 2 lists the complete information about the published methods on HPLC, LC-MS and LC-MS/MS on Huperzine A and ZT-1.

Bioanalytical related discussion

A bioanalytical method is useful tool for the quantification of the drugs from the biological matrices (plasma, serum and blood). Extraction methods play a crucial role for the estimation of drugs from these matrices. A good bioanalytical method defined as it is free from other interference substances such as salts, proteins and phospholipids except our analyte of interest. A proper bioanalytical method should be free from endogenous substances like salts, proteins and phospholipids etc.

Sample preparation

To eliminate endogenous components from the matrix, a better sample cleaning process is required. To obtain a better sensitivity and reproducibility the biological matrix should be extracted with an organic solvent(s). For the extraction of huperzine A alone or along with ZT-1 from the biological matrix most of the researchers have used ethyl acetate alone [15-17,18-22] or mixture of other solvent(s) like n-hexane:DCM:2-propanol and $CHCl_3$:IPA combination [19,20,23-25] or ethyl acetate with isopropanol [20]. Apart from these methodologies Pan et al. have adopted on-line column clean-up process to remove the endogenous components present in the biological sample(s), thus providing the interference free samples for analysis for the quantification [16,17]. Li et al. have used single step liquid-liquid extraction of rat blood samples with ethyl acetate and isopropanol mixture (containing internal standard) and vortex mixed, centrifuged, evaporated and

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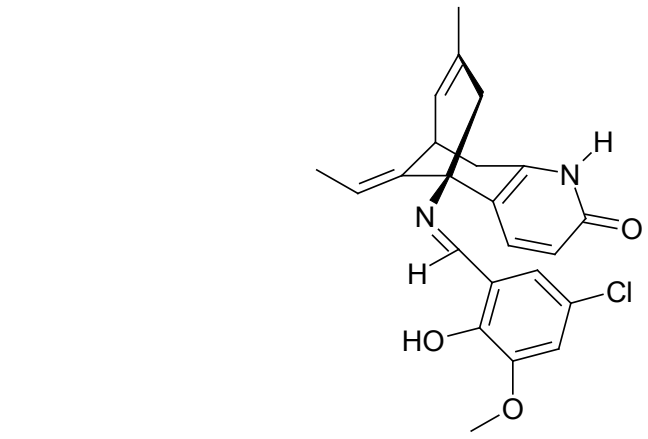
Analyte(s)	Samples processing details	Chromatographic conditions	Validation parameters	Applicable conclusions	Reference
Huperzine A	Matrix: 1 mL of human plasma. Extraction: To an aliquot of plasma, 20 µL of IS solution and 100 µL of 100 mM NaOH were and vortexed briefly. To this 5 mL of ethyl acetate was added, vortex mixed for 3 min and centrifuged for 10 min at 4000 rpm. The supernatant was evaporated to dryness under stream of nitrogen at 50°C. The residue was reconstituted with 100 µL of mobile phase. Internal standard: Trimetazidine (0.1 µg/mL in MeOH)	System: LC-ESI-MS/MS by MRM in positive mode. Column: Zorbax SB-C_{18} (100 mm × 2.1 mm, 3.5 µm) maintained at 25°C. Mobile phase: isocratic mixture comprising 10 mM ammonium acetate: MeOH: 0.1% formic acid (18:82:0.1, v/v/v) at a flow rate of 0.2 mL/min. Injection volume: 20 µL. Mass spectrometric detection: huperzine A: *m/z* 243.1 → 210.1 and IS: *m/z* 267.2 → 166.0. Retention time: ~2.0 ± 0.2 min for huperzine A and IS. Total run time: ~3.0 min	Regression type: linear fit with weighing factor (1/*x*). Calibration range: 0.01-4.0 ng/mL. Recovery: 85.2, 81.8, 85.7 and 89.2% at LLOQC, LQC, MQC and HQC, respectively. Selectivity: no endogenous interference at retention time of analyte and IS evaluated from six different sources of human blank plasma samples. Matrix effect: no significant matrix effect was observed after analyzing five different lots of human plasma. The % matrix effect was found to be 101.1-110.6. Accuracy and precision: within- and between-day accuracy (% RE) ranged from -13.0 to 0.4 and -10.2-8.2%, respectively; whereas the within- and between-day precision (% RSD) ranged from 3.78-9.22 and 7.35-11.57%, respectively. Stability: Stable under a battery of tests viz., room temperature (8 h), three F/T cycles and long term stability for 28 days at -20°C	The validated method successfully applied to PK study to estimate plasma concentrations of huperzine A in human volunteers (n=20) following oral administration of 0.1 mg tablet	[15]
Huperzine A	Matrix: 1 mL of human plasma. Extraction: To an aliquot of plasma, 20 µL of IS solution and 100 µL of 0.1 mM NaOH were added and vortexed briefly. To this 5 mL of ethyl acetate was added, vortex mixed for 3 min and centrifuged for 10 min at 4000 rpm. A 4 mL supernatant was separated and evaporated to dryness under stream of nitrogen at 50°C. The residue was reconsti-tuted with 400 µL of mobile phase. Internal standard: pseudoephedrine HCl (101.2 ng/mL in MeOH).	System: LC-ESI-MS/MS by positive mode. Column: Lichrospher C_{18} (150 mm × 4.6 mm, 5 µm) maintained at 25°C. Mobile phase: isocratic elution consists of MeOH:0.2% formic acid (85:15, v/v) at a flow rate of 0.7 mL/min. Injection volume: 20 µL. Mass spectrometric detection: *m/z* 243 → 226 and *m/z* 166 → 148 for analyte and IS, respectively. Retention time: ~2.28 and 2.32 min for huperzine A and IS, respectively. Total run time: ~4 min	Regression type: linear fit with weighing factor (1/x). Calibration range: 0.0508-5.08 ng/mL (*r*: 0.9998). Recovery: 83.6 ± 2.5, 73.6 ± 1.0 and 82.3 ± 3.9% at LQC, MQC and HQC, respectively. Selectivity: No endogenous interference at the retention time of analyte and IS. Matrix effect: No significant matrix effect was observed. Accuracy and precision: within- and between-day precision (% RSD) values were 2.18-5.47and 1.58-3.90%, respectively. Within - and between-day accuracy (% RE) values were -2.97 to 6.89 and -5.0 to 2.96%, respectively. Stability: Stable under the following tests viz. short-term stability (room temperature 4 h), auto-sampler stability (10 h), three F/T cycles and long-term stability (14 days at -20°C)	A sensitive method was developed for the determination of Huperzine A in human plasma following oral administration of 0.2 mg of tablet to Chinese volunteers	[16]

Huperzine A	Matrix: 0.1 mL of rat plasma. Extraction: Following addition of 10 µL of IS solution to an aliquot of 100 µL of rat plasma it was mixed and to it 20 µL of TCA was added. Vortex mixed for 1 min and centrifuged at 6154.5 g for 10 min. The supernatant was injected into CS-LC-MS. Following addition of 5 µL of IS solution to an aliquot of 35 µL of CSF were added. Vortex mixed for 1 min and the supernatant was injected into CS-LC-MS. Internal standard: Huperzine B (500 and 200 ng/mL in water for plasma and CSF, respectively).	System: column switching-LC-ESI-MS by SIM mode. Column: a pre-column Zorbax SB-C_{18} (12.5 mm × 4.6 mm, 5 µm) coupled to Zorbax SB-C_{18} (150 mm × 2.1 mm, 3.5 µm) was maintained at 35°C. Mobile phase: isocratic elution with column switching consists of solvent A (water containing 5 mM ammonium formate with 0.03% diethyl amine) and solvent B (ACN) (30:70, v/v) at a flow rate of 0.15 mL/min. Pre-treatment for the column 0.5 mL/min flow rate was used. Injection volume: 50 µL for plasma and 30 µL for CSF sample, respectively. Mass spectrometric detection: Huperzine A: *m/z* 243.0 and IS: *m/z* 257.0. Retention time: ~9.6 and 11.5 min for huperzine A and IS, respectively. Total run time: ~14 min	Regression type: linear fit with weighing factor (1/x). Calibration range: 0.5-500 ng/mL for plasma and 0.5-200 ng/mL for CSF (r>0.99 for both matrices). Recovery: (106.7 ± 4.14, 94.7 ± 2.85 and 99.8 ± 1.84)% at LQC, MQC and HQC, respectively for huperzine A and 102.2 ± 2.00% for IS. Selectivity: No endogenous interference at the retention time of analyte and IS. Matrix effect: No significant matrix effect was observed after analyzing different lots of plasma and CSF. % ME was found to be 84.3-89.9 and 86.2-97.6% for plasma and CSF, respectively. Accuracy and precision: accuracy values were found to be within ±7.91%. Within- and between-day precision (% RSD) values were <2.20 and <6.67% for plasma. Accuracy values were found to be within ±7.55%. Within- and between-day precision (% RSD) values were <0.822 and <6.46% for CSF. Stability: Stable in auto-sampler (24°C), after four F/T cycles, and long term for 60 days at -20°C for plasma and CSF whereas in case of F/T stability only three cycles was done for CSF.	Authors have used a column switching for the estimation of huperzine A from rat plasma and CSF. The validated application has been shown for estimation of huperzine A in rat plasma and CSF following nasal and *i.v* administration in rat.	[17]
Huperzine A	Matrix: 1 mL human plasma. Extraction: to an aliquot of plasma, 100 µL of IS solution mixed and vortexed for 15 s then add 100 µL of 1M NaOH, vortex-mixed and extracted with 5 mL ethyl acetate vortex for 5 min and kept aside for 1 min for better extraction. The contents were centrifuged for 15 min at 4000 rpm and the clear organic layer was dried under gentle stream of nitrogen at 40°C. The residue was reconstituted in 100 µL of MeOH with vortexing for 1 min. Again centrifuged this solution at 12,000 rpm for 5 min. Internal standard: Codeine phosphate (50 ng/mL in MeOH).	System: LC-ESI-MS/MS by MRM in positive mode. Column: Diamonsil C_{18} (150 mm × 4.6 mm, 5 µm) at ambient temperature. Mobile phase: isocratic mobile phase comprising MeOH: 1% formic acid (60:40, v/v) at a flow rate of 1.0 mL/min. Injection volume: 40 µL. Mass spectrometric detection: *m/z* 243 → 210 and *m/z* 300 → 199 for analyte and IS, respectively. Retention time: ~1.36 and 1.30 min for huperzine A and IS, respectively. Total run time: 2 min.	Regression type: linear fit with weighing factor ($1/x^2$). Calibration range: 0.126-25.2 ng/mL (r^2: 0.9978). Recovery: 85.7, 83.5, 82.9 and 81.6% at LLOQC, LQC, MQC and HQC, respectively for huperzine A. Selectivity: No endogenous interference at retention time of analyte and IS evaluated from six sources of human blank plasma samples. Matrix effect: No significant matrix effect was observed. Accuracy: Within- and between day accuracy were found to be within acceptable limits. Precision: Within- and between-day precision (% RSD) values were 3.39-13.68 and 0.66-14.4%, respectively. Stability: Stable under the following tests viz. stock solution stability (30 days at 4°C), auto-sampler stability (24 h), three F/T cycles and long-term stability (40 days at -20°C).	A sensitive method was developed for the determination of Huperzine A in human plasma.	[18]

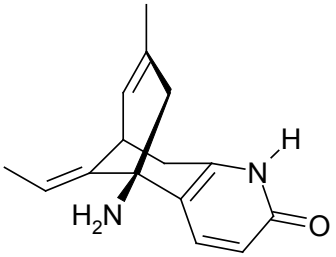
Huperzine A	Matrix: 500 µL dog plasma. Extraction: Following spiking of 100 µL of IS solution, 100 µL of MeOH and 100 µL of 1 M of NaOH into an aliquot of plasma the contents were vortex-mixed and extracted with 4 mL of *n*-hexane:DCM:2-propanol (300:150:15, v/v/v) vortex for 15 min. Following centrifugation for 5 min at 3000 *g* the organic layer was evaporated to dryness under gentle sream of nitrogen at 40°Cand the residue was reconstituted in 100 µL of mobile phase. Internal standard: huperzine B (100 ng/mL in MeOH).	System: LC-ESI-MS/MS by MRM in positive mode. Column: Nucleosil C_{18} (50 mm × 4.6 mm, 5 µm) maintained at ambient temperature. Mobile phase: isocratic mobile phase comprising ACN:MeOH:10 mM ammonium acetate (35:40:25, v/v/v) at a flow rate of 0.4 mL/min. Injection Volume: 10 µL. Mass spectrometric detection: huperzine A: *m/z* 243.4 → 210.2 and IS *m/z* 257.4 → 226.2, respectively. Retention time: ~1.64 and 1.69 min for huperzine A and IS, respectively. Total run time: ~2.0 min.	Regression type: Linear fit with weighing factor ($1/x^2$). Calibration range: 0.05-20 ng/mL (r^2: 0.9991). Recovery: 77.1 ± 4.0, 85.7 ± 2.8 and 86.3 ± 2.2% at LQC, MQC and HQC, respectively. Selectivity: No endogenous interference at retention times of analyte and IS. Matrix effect: no significant matrix effect was observed. Accuracy and precision: Accuracy values (% RE) was found to be <2.25; whereas the within- and between day precision (% RSD) ranged between 2.44-3.72 and 3.16-5.33%. Stability: Stable under battery of tests viz. stock solution stability (60 days at 4°C), auto-sampler stability (24 h) and long term stability 90 days at -20°C.	The validated method was successfully employed in a pre-clinical PK study to quantitate huperzine A concentrations after an *i.m* dosing a sustained-release formulation (10 µg/kg) for 15 days in Beagle dogs.	[19]
ZT-1 and huperzine A	Matrix: 0.2 mL of rat blood. Extraction: To an aliquot of blood, 100 µL of buffer (0.4 M NaOH:0.2 M Na_2HPO_4 : 26.9:50, v/v; pH 12) and 10 µL of IS solution were and vortexed briefly. To this mixture of ethyl acetate:IPA (950:50, v/v) was added, vortex mixed for 3 min each and centrifuged for 3 min at 2000 g and 16060 g. The supernatant was evaporated to dryness under stream of nitrogen at 35°C. The residue was reconsti-tuted with 150 µL of mobile phase (MeOH: H_2O (50:50, v/v) containing 0.05% of $HCOONH_4$. Internal standard: huperzine B (100 ng/mL in water).	System: LC-ESI-MS/MS by MRM in positive mode. Column: Supelcosil LC_{18} (100 mm × 3.0 mm, 5 µm) maintained at 30°C. Mobile phase: linear gradient mixture comprising solvent A [MeOH:H_2O (10:490, v/v containing 0.05% $HCOONH_4$)]: solvent B [MeOH:H_2O (450:50, v/v containing 0.05% $HCOONH_4$)] (45:55, v/v) at a flow rate of 0.2 mL/min. Injection volume: 90 µL. Mass spectrometric detection: *m/z* 243 → 226, *m/z* 413 → 226 and *m/z* 257 → 240, for huperzine A, ZT-1 and IS, respectively. Retention time: (~7.2 ± 0.2, ~12.2 ± 0.2 and ~8.3 ± 0.2) min for huperzine A, ZT-1and IS, respectively. Total run time: ~18.0 min.	Regression type: linear fit with weighing factor ($1/x$). Calibration range: 0.12-30 and 0.51-125 ng/mL for ZT-1 and huperzine A, respectively, (r>0.99 for both analytes). Recovery: >88% for both huperzine A and ZT-1 and 85% for IS. Selectivity: No endogenous interference at retention time of analyte and IS. Matrix effect: No significant matrix effect was observed. Accuracy and precision: Within- and between-day precision (% RSD) ranged from -8.4 to10.3 and 8.3-9.8%, respectively for ZT-1; whereas the within- and between-day precision (% RSD) ranged from 7.3-10.4 and 7.8-10.2%, respectively for huperzine A.	The validated method successfully applied to pharmacokinetic (PK) study to estimate blood concentrations of ZT-1 and huperzine A in rat following *i.g* or *i.v* administration of 0.5 and 0.1 mg/kg.	[20]
Huperzine A	Matrix: 3 mg of tissue homogenate from clubmoss *Huperzia squarrosa*. Extraction: extract freeze-dried tissue with 2 mL of aqueous acetic acid solution (2% v/v containing 2.7 µg/mL of atropine) by vigorously vortexed for 10 min followed by ultrasonication for 30 min at 23°C. This mixture was centrifuged for 2 min at 2500 g. The supernatant was extracted again with 2 mL of 2% acetic acid followed by 3 mL of $CHCl_3$. After exitraction $CHCl_3$ layer was discarded and the aqueous layer pH was adjusted to 11 using 9 M NH_3. The basic solution again extracted twice with 3 mL of $CHCl_3$. The supernatant ($CHCl_3$ layer) was evaporated to dryness under stream of nitrogen. The residue was reconstituted with 300 µL of MeOH and filtered by 0.45 µm PTFE syringe. Internal standard: atropine (2.7 µg/mL in 2% acetic acid solution).	System: UHPLC-APCI- MS QTOF in positive mode. Column: Zorbax Eclipse Plus C_{18} (50 mm × 2.1 mm, 1.8 µm) maintained at 60°C. Mobile phase: linear gradient mixture comprising solvent A (0.2% aqueous acetic acid): solvent B (0.2% acetic acid in MeOH) at a flow rate of 1.3 mL/min. Injection volume: 2 µL. Mass spectrometric detection: *m/z* 243 → 226for huperzine A. Retention time: ~0.62 min for huperzine A. Total run time: ~4.0 min.	Regression type: Linear fit with weighing factor ($1/x^2$). Calibration range: 0.01-50 µg/mL for huperzine A, (r^2>0.99 for both analytes). Recovery: 99.5% for huperzine A. Selectivity: No endogenous interference at retention time of analyte and IS. Matrix effect: No significant matrix effect was observed in *H. squarrosa*.	This method was successfully applied to estimate huperzine A concentrations in green-house grown *H. squarrosa* sporophytes.	[21]

Huperzine A	Matrix: 1 mL of human plasma. Extraction: To an aliquot of plasma, 100 µL of buffer (0.1 M NaOH) and 20 µL of IS solution were added and vortexed briefly for 30 s. To this 6 mL of ethyl acetate was added, vortex mixed for 5 min and centrifuged for 10 min at 10,000 rpm. The supernatant was evaporated to dryness under stream of nitrogen at 35°C. The residue was reconstituted with 100 µL of ACN and vortex mixed for 30 s followed by centrifugation for 5 min at 10,000 rpm. Internal standard: codeine phosphate (15 ng/mL in MeOH).	System: LC-ESI-MS/MS by MRM in positive mode. Column: Altima HP HILIC (150 × 2.0 mm, 3 µm) for HILIC separation. Gemini C_{18} (150 × 2.0 mm, 3 µm) for RPLC separation. Both of these conditions were maintained at 35°C. Mobile phase: an isocratic flow rate comprising 5 mM ammonium formate and ACN (87:13, v/v) at a flow rate of 0.2 mL/min for HILIC elution. An isocratic flow rate comprising 0.5% formic acid and MeOH (60:40, v/v) at a flow rate of 0.2 mL/min for RPLC elution. Injection volume: 20 µL. Mass spectrometric detection: *m/z* 243 → 226 and *m/z* 300 → 199 for huperzine A and IS, respectively. Retention time: ~11.6 ± 0.2 and ~13.2 ± 0.2 min for huperzine A and IS, respectively. Total run time: ~16.0 min.	Regression type: Linear fit with weighing factor ($1/x^2$). Calibration range: 0.05-5.0 for huperzine A, (r>0.99 for both analytes). Recovery: >86% for huperzine A and 87% for IS. Selectivity: No endogenous interference at retention time of analyte and IS. Matrix effect: No significant ME was observed in HILIC condition but ME was observed (ME: 14.8-79.4%) in RPLC-MS/MS. Accuracy and precision: Within- and between-day precision (% RSD) ranged from 1.6-7.2%; whereas the within- and between-day precision (% RE) ranged from 0.8-5%, respectively for huperzine A. Stability: Stable under battery of tests viz. three F/T cycles, room temperature (6 h), auto-sampler stability (24 h) and long term stability 42 days at -80°C.	The validated method successfully applied to pharmacokinetic (PK) study to estimate plasma concentrations of huperzine A in humans following oral administration of 0.2 mg/kg.	[22]

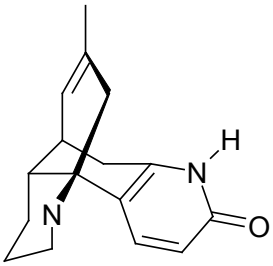
Table 1: Summary validation of various published LC-MS/MS methods for ZT-1 and huperzine A. ACN: Acetonitrile; $CHCl_3$: Chloroform; CSF: Cerebrospinal Fluid; CS-LC-MS: Column Switching Liquid Chromatography Mass Spectrometry; DCM: Dichloromethane; ESI: Electro Spray Ionization; F/T: Freeze Thaw; HCl: Hydrochloric Acid; HQC: High Quality Control; $HCOONH_4$: Ammonium Formate; HILIC: Hydrophilic Interaction Chromatography; AF: Ammonium Formate; IPA: Isopropanol; IS: Internal Standard; i.g.: Intragastric; i.v.: Intravenous; LLOQC: Lower Limit of Quality Control; LQC: Low Quality Control; ME: Matrix Effect; MQC: Medium Quality Control; MRM: Multiple Reaction Monitoring; MeOH: Methanol; Na_2HPO_4: Disodium Phosphate; NaOH: Sodium Hydroxide; NH_3: Ammonia; PTFE: Polytetrafluoroethylene; PK: Pharmacokinetic; RE: Relative Error; RPLC: Reverse Phase Liquid Chromatography; RSD: Relative Standard Deviation; SIM: Selected Ion Monitoring; TCA: Trichloroacetic Acid.



ZT-1



Huperzine A



Huperzine B

Figure 1: Structural representation of ZT-1, Huperzine A and Huperzine B.

Analytes(s)	Samples processing details	Chromatographic conditions	Validation parameters	Applicable conclusions	Reference
Huperzine A	Matrix: 200 µL of rat plasma. Extraction: to an aliquot of plasma 10 µL of IS and 100 µL of borax-sodium carbonate buffer (pH 11.8) were added, vortex mixed and to this add 2 mL of $CHCl_3$ vortex mixed for 5 min followed by centrifugation for 10 min at 3000 *g*. The supernatant was evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted in 50 µL of mobile phase. Internal standard: (*S*)-9, 10-difluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-de] [1,4]benz-oxazine-6-carboxylic acid methyl ester (2.0 µg/mL in MeOH).	System: HPLC with fluorescence detector. Column: Kromasil C_8 (150 mm × 4.6 mm, 5 µm) maintained at 40°C. Mobile phase: MeOH:water:TEA (45:55:0.05, v/v/v) with an isocratic elution at a flow rate of 1 mL/min. Injection volume: 20 µL. Detection: Excitation and emission detection was set 310 and 370 nm, respectively. Retention time: ~7.0 and 8.9 min for huperzine A and IS, respectively. Total run time: ~12 min.	Regression type: linear fit with weighing factor (1/*x*). Calibration range: 2.5-250 ng/mL (*r*: 0.999). Limit of detection: 2.5 ng/mL. Recovery: 84.6 ± 8.8, 79.1 ± 11.2 and 73.5 ± 1.3% at LQC, MQC and HQC, respectively. Selectivity: No endogenous interference at the retention time of the analyte and IS. Precision: Within- and between-day (% RSD) values are found to be 4.9-10.1% and 9.2-14.1%. Stability: Stable for 30 days at-20°C.	The validated method was used to quantitate the plasma concentrations of huperzine A following intranasal administration to rats at 50 µg dose also this method is suitable for PK studies.	[23]
ZT-1 and huperzine A	Matrix: 2.0 mL of rat/dog plasma. Extraction: prepared the ZT-1 standard solution with MeOH and dried with nitrogen, then add 2.0 mL plasma into it. To this add 5 mL of $CHCl_3$: IPA (9:1, v/v) and vortex mixed for 2 min followed by centrifugation for 5 min at 15,000 rpm. Then 4 mL supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 160 µL of MeOH. Internal standard: No IS was used.	System: HPLC with UV detector. Column: Nucleosil C18 (250 mm × 4.6 mm, 5 µm) maintained at 35°C. Mobile phase: MeOH: 1mM ammonium acetate (30:70, v/v) at a flow rate of 0.7 mL/min. Injection volume: 50 µL. Detection: λ_{max} set at 313 nm. Retention time: ~18.7 and 14.4 min for ZT-1 and huperzine A, respectively. Total run time: ~26 min.	Regression type: Linear fit with weighing factor (1/*x*). Calibration range: 0.02-2.0 and 0.02-5.0 nM/mL for ZT-1 and huperzine A, respectively. The correlation coefficient (r) for ZT-1 and huperzine A was found to be 0.997 and 0.999, respectively. Recovery: (107.8 ± 9.0, 91.9 ± 2.4 and 90.0 ± 2.1%) for ZT-1 at LQC, MQC and HQC, respectively. (98.9 ± 1.8, 101.7 ± 1.8 and 96.6 ± 1.8%) for huperzine A at LQC, MQC and HQC, respectively. Selectivity: No endogenous interference at the retention time of the analyte and IS. Accuracy and Precision: within-day values are found to be 2.0 to 9.5% and % RE was found to be -3.5 to 5.0 for ZT-1. Within- and between-day precision values are found to be 4.5-14.3% and 2.0-15.0% and Within- and between-day % R.E. was found to be 2.0 to 5.0 and 0.0 to 2.4 for huperzine A, respectively.	The validated method was used to quantitate ZT-1 and Huperzine A in rat and dog plasma following adminis-tration of 5 and 2.5 mg/kg oral and *i.g.* dose, respectively.	[24]
Huperzine A	Matrix: 2.5 mL of dog serum. Extraction: an aliquot of serum was made alkaline (pH 9.5) by adding 1.0 mL of borax-sodium carbonate buffer (pH 10.8-11.2), then 35 µL of IS and 3.0 mL $CHCl_3$:IPA (95:5, v/v) were added, vortex mixed for 2 min and centrifuged for 10 min at 3000 g. Supernatant was removed and evaporated to dryness under nitrogen at 50°C. Extraction procedure was carried at twice. The residue was dissolved in 100 µL of mobile phase centrifuged for 2 min at 2000 *g*. Internal standard: Mebendazole (0.5 µg/mL in water)	System: HPLC with UV detector. Column: Shimpack CLC-ODS (150 mm × 4.6 mm, 5 µm) maintained at 35°C. Mobile phase: isocratic elution comprising MeOH: water:glacial acetic acid (50:48.5:1.5, v/v/v) using sodium dodecyl sulfonate as an ion pairing agent at a flow rate of 1 mL/min. Injection volume: 50 µL. Detection: λ_{max} set at 313 nm. Retention time: ~12.9 and 10.0 min for huperzine A and IS, respectively. Total run time ~14 min.	Regression type: Linear fit with weighing factor (1/*x*). Calibration range: 1-12 ng/mL (*r*:>0.999). Limit of detection: 0.5 ng/mL. Selectivity: no endogenous interference at the retention time of the analyte and IS. Recovery: (76.0 ± 10.2, 72.1 ± 2.3 and 74.0 ± 4.0%) at LQC, MQC and HQC, respectively. Precision: Within- and between-day RSD values were 3.0-12.8% and 5.8-10.8%, respectively. Stability: Stable for 24 h at room temperature and 32 days at -20°C.	This method proved to be useful to characterize the PK study of huperzine A transdermal patches in Beagle dogs.	[25]

Table 2: Summary validation of various published HPLC methods for ZT-1 and huperzine A. $CHCl_3$: Chloroform; HPLC: High Performance Liquid Chromatography; IS: Internal Standard; i.g.: Intragastric; MeOH: Methanol; RE: Relative Error; RSD: Relative Standard Deviation; UV: Ultra Violet; LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control; TEA: Triethanolamine.

reconstituted in mobile phase [20]. In other reported liquid-liquid extraction methods for the estimation of huperzine A in the biological sample the pH was made to basic because of its pKa value of 7.7 before extraction process [15,18,19]. Some researchers have reported making the plasma pH to 9-12 by adding borax-sodium carbonate buffer to get better recovery in biological matrix [23,24]. Cuthbertson et al. adopted the quantification of Huperzine A from the microscale extraction of clubmoss Huperzia squarrosa using liquid extraction by using $CHCl_3$ with 99.95% recovery. The detection limit was 20 pg by using UHPLC-QTOF-MS instrument [21].

Internal standard (IS) selection

Selection of a proper IS a critical part of the bioanalysis. An ideal IS must meet same extraction recovery, ionization response and almost similar molecular weight of the analyte. Stable Isotopically Labeled (SIL) internal standards give accurate results compared to deuterate IS [26]. Yue et al. reported using a (S)-9, 10-difluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid methyl ester as IS for quantification huperzine A in rat plasma on HPLC with fluorescence detector [23]. Mebendazole was used as IS for the quantification huperzine A in dog plasma on HPLC with UV detector [25]. Some researchers Wei et al. [17] and Wang et al. [19] have been using huperzine B (Figure 1) as an IS to avoid difficulties to measure drug levels on LC-MS/MS [16,18] and Li et al. have also used huperzine B as an IS in quantifying ZT-1 and huperzine A from rat blood [20]. Codeine phosphate [15,22], trimetazidine diHCl [16] and pseudoephedrine HCl [18] were other ISs used while quantifying huperzine A from plasma. Tropane alkaloid atropine was used as an IS for the quantification of huperzine A from plant cuttings of *H. saquarrosa* [21]. Wei et al. [24] did not use any IS for the quantification of ZT-1 and huperzine A from rat/dog plasma.

Matrix effect

It is defined as an alteration or interference in the sensitivity of an analyte of the sample. For low volatile compounds ionization suppression is a common practice, because of this matrix effect will occur and it can be reduced by doing simple dilution [27]. To get a better recovery and free from sample interference three C_{18} columns were used for column switching process [17]. Samples can be basified with sodium hydroxide to get rid of ion suppression followed by increasing the sensitivity and recovery of analyte [15]. Two chromatographic separation methods (HILIC and RPLC) were optimized for Wei et al. for the separation of huperzine A in human plasma, RPLC method showed significant levels of matrix effect [22].

Chromatography

A variety of columns were used, with most of the methods a temperature ranging from 25-40°C has been used to achieve the good chromatographic separation of huperzine A in human [15,16,18,22] rat [17,23,24] and dog plasma [19,25] and huperzine A with ZT-1 from rat blood [20] and dog/rat plasma [24]. All the methods are reported by LC-MS or LC-MS/MS used by C_{18} columns for better peak resolution by using either, isocratic [16-19,22-25] or gradient [20,21] or HILIC [22] separation with a flow rate range between 0.2-1.0 mL/min. Pan et al. [17] used an automated column switching technique with three C_{18} columns (Agilent SB-C_{18}, Zorbax SB-C_{18} and C_{18} guard column) to get a better recovery and reduce the interference from the biological matrix. Also the initial flow rate was 0.15 mL/min which gradually increased by 0.5 mL/min followed by an isocratic separation of huperzine A from the plasma [17]. In HPLC methods, reverse-phase mode C_{18} [24,25] and normal phase mode C_8 [23] columns were used for validation purpose. Generally the column length ranged from 50-250 mm and the external diameter was 2.1-4.6 mm with a particle size ranging from 3.5-5 µm.

Detection

Major detection methods were mass spectrometry [15-20,22] UHPLC-QTOF-MS [21], HPLC with fluorescence detection [23] and ultra violet detection [24,25]. In current scenario, by using a mass detector is most widely used because of its high sensitivity and selectivity for the detection and estimation from the biological matrices. Currently LC-MS/MS is a popular analytical tool for the quantitative bioanalysis of drugs in pharmaceutical industry due to its superior sensitivity and selectivity. Ye et al. [25] reported anion-paring reverse phase method on dog serum by adjusting the pH to 9.5 by adding borax-sodium carbonate buffer (pH 10.8-11.2). These basic conditions are best suitable for ionized compounds. Adding ionic compounds (sodium dodecyl sulfonate) to the mobile phase to increase the creation of ion pairs with charged analyte to improve the thus the sensitivity of the molecule will be improved. Though Huperzine A is having chromophore at different sites majority of the HPLC methods were reported by ultra violet detection [24,25] at a maximum absorption at 313 nm. An excitation wavelength of 310 nm and emission wavelength of 370 nm was reported by Yue et al. by using a fluorescence detector [23].

Fragmentation of huperzine A mainly happened in the amino group. Li et al. [20] have reported a product ion of m/z 226 and $[M^+Na]^+$ ions reported by Zou et al. and Wang et al. respectively [15,19]. Upon fragmentation there is a sequential loss of m/z 243, 226 and 210 was reported by Zou et al.; Li et al.; Wang et al.; Li et al.; Cuthbertson et al.; and Wei et al. respectively [15,18-22] and a Selective Ion Monitors (SIM) method was reported for the quantification of huperzine A in rat plasma and cerebrospinal fluid [17]. In MRM mode, almost all the methods are reported quantitation using by a product ion of m/z 226.

Discussion

The bioanalysis of ZT-1 and huperzine A gives many challenges during method development for bioanalytical scientists. Based on the review of the literature, following perspectives may consider for the development of bioanalytical methods for ZT-1 and huperzine A.

Huperzine A consists of one ionizable carboxylic group and amino groups with pKa value of 7.7; therefore pH adjustment in the mobile phase is very important to ensure adequate elution of compound of interest from the column. To give better sensitivity or ionization it is important to basify the biological samples.

Due to the presence of chromophoric groups in the structure ideally UV detector should be selected. Selectivity considerations need to be carefully evaluated.

Conclusion

Huperzine A is an important alkaloid to treat various indications of neurodegeneration especially Alzheimer's disease and also used in the treatment of rheumatism, to relax muscles and improve blood circulation. However, several methods are reported that have independently addressed many of the difficulties associated with the separation and detection of huperzine A. All the reported methods by HPLC, the linearity range from 1-250 ng/mL and very sensitivity sensitive methods compared to HPLC by LC-MS or LC-MS/MS ranging from 0.01-500 ng/mL, respectively. These sensitive LC-MS/MS methods are captured a clear elimination phase after post-dosing with a narrow dose range of 0.1-0.5 mg/kg.

Conflicts of Interest

The authors have no conflicts of interest relevant to the ideas and/or contents of the manuscript.

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